Biochemical Journal Immediate Publication. Published on 28 May 2004 as manuscript BJ20040637

# Inositol trisphosphate analogues selective for types I and II inositol trisphosphate receptors exert differential effects on vasopressin-stimulated $Ca^{2+}$ inflow and $Ca^{2+}$ release from intracellular stores in rat hepatocytes

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**Short Title: Inositol trisphosphate receptors and store-operated $Ca^{2+}$ channels**

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**Biochemical Journal Immediate Publication. Published on 28 May 2004 as manuscript BJ20040637**



## SUMMARY

Previous studies have shown that adenophostin A is a potent initiator of the activation of store-operated $Ca^{2+}$ channels (SOCs) in rat hepatocytes, and have suggested that, of the two subtypes of inositol trisphosphate ($Ins(1,4,5)P_3$) receptor predominantly present in rat hepatocytes (types 1 and 2 ($Ins(1,4,5)P_3R_1$ and $Ins(1,4,5)P_3R_2$)), $Ins(1,4,5)P_3R_1$s are required for SOC activation. We compared the abilities of $Ins(1,4,6)P_3$ (higher apparent affinity for $Ins(1,4,5)P_3R_1$) and $Ins(1,3,6)P_3$ and $Ins(1,2,4,5)P_4$ (higher apparent affinities for $Ins(1,4,5)P_3R_2$) to activate SOCs. The $Ins(1,4,5)P_3$ analogues were microinjected into single cells together with fura-2, and dose-response curves for the activation of $Ca^{2+}$ inflow and $Ca^{2+}$ release from intracellular stores obtained for each analogue. The concentration of $Ins(1,4,6)P_3$ which gave half-maximal stimulation of $Ca^{2+}$ inflow was substantially lower than that which gave half-maximal stimulation of $Ca^{2+}$ release. By contrast, for $Ins(1,3,6)P_3$ and $Ins(1,2,4,5)P_3$, the concentration which gave half-maximal stimulation of $Ca^{2+}$ inflow was substantially higher than that which gave half-maximal stimulation of $Ca^{2+}$ release. The distribution of $Ins(1,4,5)P_3R_1$ and $Ins(1,4,5)P_3R_2$ in rat hepatocytes cultured under the same conditions as those employed for the measurement of $Ca^{2+}$ inflow and release was determined by immunofluorescence. $Ins(1,4,5)P_3R_1$s were found predominantly at the cell periphery whereas $Ins(1,4,5)P_3R_2$s were found at the cell periphery, the cell interior and nucleus. It is concluded that the idea that a small region of the ER enriched in $Ins(1,4,5)P_3R_1$ is required for the activation of SOCs is consistent with the present results for hepatocytes.

*Keywords:* Store-operated $Ca^{2+}$ channels, endoplasmic reticulum, inositol trisphosphate receptors.

*Abbreviations used:* $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $Ca^{2+}_o$, extracellular $Ca^{2+}$; $Ins(1,4,5)P_3R$, $InsP_3$ receptor; ER, endoplasmic reticulum; $[Ca^{2+}]_{cyt}$, cytoplasmic $Ca^{2+}$ concentration.

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## INTRODUCTION

Store-operated $Ca^{2+}$ channels (SOCs) in the plasma membrane are required for regulation of the cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$) and re-filling the endoplasmic reticulum (ER) $Ca^{2+}$ stores in hepatocytes and in other non-excitable cells, and in some excitable cells [1,2]. Several studies have shown that SOCs are required for the maintenance of agonist-induced oscillations in $[Ca^{2+}]_{cyt}$ [3,4] and there is evidence that SOCs are more effective in re-filling the ER than non-selective cation channels [5]. The activation of SOCs is initiated by a decrease in the concentration of $Ca^{2+}$ in the ER induced by the action of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) at $Ins(1,4,5)P_3$ receptors ($Ins(1,4,5)P_3R$) and by $Ca^{2+}$ at ryanodine receptors [1,2]. There is some evidence which suggests there may be a direct interaction (conformational coupling) between some $Ins(1,4,5)P_3Rs$ and some putative SOCs or other types of plasma membrane $Ca^{2+}$ channels (reviewed in [1,2]). Evidence that the activation of SOCs involves, or requires, a specific region of the ER close to the plasma membrane in the vicinity of SOCs [6-9] and/or continuity of the whole ER [10,11] has been reported. While there have been numerous experiments designed to determine the relationship between the degree to which $Ca^{2+}$ in the ER is decreased and the activation of SOCs, and the location of this decrease in ER $Ca^{2+}$ [12,13], no clear answers have so far been obtained.

Rat hepatocytes *in situ* are polarised epithelial cells with clearly-defined canalicular, basal and basolateral membrane regions (reviewed in [14]). Freshly-isolated rat hepatocytes lose much of this polarity but after culture for a few hours begin to regain some polarity [15]. Normal rat hepatocytes possess predominantly $Ins(1,4,5)P_3R_1$ and $Ins(1,4,5)P_3R_2$ with very little type 3 $Ins(1,4,5)P_3$ receptor [16,17]. $Ins(1,4,5)P_3R_2$ predominate, and may be responsible for the generation of $[Ca^{2+}]_{cyt}$ waves emanating from the canalicular region [17]. There is evidence that $Ins(1,4,5)P_3R_1s$ are associated with a subregion of the ER which is close to the plasma membrane and attached to the peripheral F-actin [18,19].

The results of previous studies with rat hepatocytes, which employed adenophostin A, an $Ins(1,4,5)P_3$ analogue with a very high affinity for $Ins(1,4,5)P_3R$, 2-hydroxyethyl-α- D-glucopyranoside 2,3',4'-trisphosphate ($Glc(2,3',4')P_3$), and an antibody against $Ins(1,4,5)P_3R_1$ which inhibits $Ins(1,4,5)P_3R$ function, suggested that a subregion of the ER and the $Ins(1,4,5)P_3R_1$ are both required for the activation of SOCs [9]. Adenophostin A has been used to investigate the role of $Ins(1,4,5)P_3R$ and the ER in the mechanism of activation of SOCs in

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several other cell types [7,20] leading, in some studies, to the conclusion that a small sub-region of the ER is required for SOC activation [7].

The aim of the present studies was to test further the roles of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ in the activation of SOCs in rat hepatocytes. This has been done using Ins(1,4,5)$P_3$ analogues with different apparent affinities for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ [21] to initiate intracellular $Ca^{2+}$ release and $Ca^{2+}$ inflow. The intracellular locations of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ under the same primary cell culture conditions as those employed for the measurement of $Ca^{2+}$ inflow and release were also determined by immunofluorescence. The results provide evidence which indicates that a region of the ER (or another intracellular $Ca^{2+}$ store) which is enriched in Ins(1,4,5)$P_3R_1$ is involved in the activation of SOCs.

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## MATERIALS AND METHODS

### Materials

D-*myo*-Ins(1,3,6)$P_3$ [22], D-*myo*-Ins(1,4,6)$P_3$ [23], and D-*myo*-Ins(1,2,4,5)$P_4$ [24] were synthesised as described previously. D-*myo*-2-Deoxy-Ins(1,4,5)$P_3$ (AM Riley and BVL Potter unpublished) was synthesized from D-3,6-di-*O*-benzyl-4,5-*O*-(2,3-dimethoxybutane-2,3-diyl)-*myo*-inositol [25]. All ligands were prepared as their tri-ethylammonium salts and were homogenous by routine spectroscopic methods. The lyophilised form of each Ins(1,4,5)$P_3$ analogue was reconstituted in water, washed through 0.5 ml Chelex-100 resin (to replace the tri-ethylammonium cation with $Na^+$) and again lyophilised. Stock solutions were prepared by dissolving the lyophilised Ins(1,4,5)$P_3$ analogue in 125 mM KCl. Monoclonal antibodies KM1112 and KM1083 specific for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$, respectively, [26] were kindly provided by Professor K Mikoshiba, University of Tokyo. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Sigma. Nitrocellulose and PVDF membranes and ECL detection reagents were provided by Amersham. SaOS-2 human osteosarcoma cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were kindly provided by Dr T.J. McCann, Babraham Institute, Cambridgeshire, UK, and L15 mouse fibroblasts [27] by Professor K. Mikoshiba, University of Tokyo, Japan. SaOS-2 cells [28] and L15 mouse fibroblasts [29] were cultured as described previously.

### Isolation of hepatocytes and measurement of the cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$)

The isolation of hepatocytes from Hooded Wistar rats, attachment of hepatocytes to collagen-coated coverslips, the microinjection of fura-2 and Ins(1,4,5)$P_3$ analogues and measurement of the fluorescence of single hepatocytes loaded with fura-2 (using a ratiometric technique) were carried out as described previously [9]. Changes in $Ca^{2+}$ concentration are expressed as changes in the fluorescence ratio ($F_{340nm}/F_{380\ nm}$). The dilution factor for the microinjection of agents to hepatocytes was determined to be 1:75 [9] and was used to estimate the intracellular concentrations of the Ins(1,4,5)$P_3$ analogues microinjected into hepatocytes.

For estimates of the amounts of $Ca^{2+}$ released from intracellular stores and rates of $Ca^{2+}$ inflow, hepatocytes attached to collagen-coated coverslips were microinjected with fura-2 together with a given Ins(1,4,5)$P_3$ analogue, incubated for 10 min (to allow the cells to re-seal [9]), transferred to a medium containing no added $Ca^{2+}$, and the fluorescence ratios measured.

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Rates of $Ca^{2+}$ inflow in single hepatocytes were estimated following the addition of extracellular $Ca^{2+}$ ($Ca^{2+}_o$) to cells incubated in the absence of added $Ca^{2+}_o$ (the "$Ca^{2+}$ add-back" protocol) [9] from measurement of the initial rate of the $Ca^{2+}$-induced increase in $[Ca^{2+}]_{cyt}$ (expressed as change in fluorescent ratio units per min). Since the amount of $Ca^{2+}$ which accumulates in the cytoplasmic space in a $Ca^{2+}$ add-back protocol depends on both the rate of inflow across the plasma membrane and the rate of removal from the cytoplasmic space by transport into intracellular stores and the extracellular space, the initial rate of increase in $[Ca^{2+}]_{cyt}$ is a more accurate reflection of the rate of $Ca^{2+}$ inflow through SOCs than the value of the subsequent plateau in $[Ca^{2+}]_{cyt}$ (underlying assumptions discussed in [30]).

To estimate the amount of $Ca^{2+}$ released from intracellular stores by a given Ins(1,4,5)$P_3$ analogue, vasopressin (40 nM) was added to cells loaded with the Ins(1,4,5)$P_3$ analogue plus fura-2, or with fura-2 alone. The amount of $Ca^{2+}$ released by the Ins(1,4,5)$P_3$ analogue was calculated as the difference between the vasopressin-induced release of $Ca^{2+}$ measured in the absence and presence of the Ins(1,4,5)$P_3$ analogue. It has previously been shown, using EGTA to chelate extracellular $Ca^{2+}$, that the rapid increase in $[Ca^{2+}]_{cyt}$ induced by vasopressin in the presence of extracellular $Ca^{2+}$ reflects the release of $Ca^{2+}$ from intracellular stores with little contribution from $Ca^{2+}$ inflow across the plasma membrane [9]. This was confirmed in the present series of experiments by measuring vasopressin-induced $Ca^{2+}$ release (the height of the vasopressin-induced peak of $[Ca^{2+}]_{cyt}$) in the presence of $Ca^{2+}_o$ (1.5 mM) and in the presence of both $Ca^{2+}_o$ and EGTA (2 mM). Experiments were performed with cells microinjected with fura-2 plus Ins(1,4,6)$P_3$ (10 μM), Ins(1,2,4,5)$P_4$ (70 μM) and Ins(1,3,6)$P_3$ (110 μM). For each analogue there was no significant difference in the amount of $Ca^{2+}$ released in response to vasopressin (40 nM) measured in the presence and absence of EGTA. The amounts of $Ca^{2+}$ release induced by vasopressin were 0.28 ± 0.02 and 0.36 ± 0.04 (EGTA); 0.52 ± 0.05 and 0.51 ± 0.05 (EGTA); and 0.58 ± 0.04 and 0.53 ± 0.03 (EGTA) (means ± SEM, n= 8-15) for cells containing Ins(1,4,6)$P_3$, Ins(1,2,4,5)$P_4$, and Ins(1,3,6)$P_3$, respectively.

**Western blot analysis**

Tissue preparation, protein assays, SDS gels, and semi-dry blotting were performed essentially as described previously [29]. SDS-minigels (6%) were run using the Biorad Immunoblot assay kit. Standard homogenisation, Western blotting and transfer buffers were used throughout, and either nitrocellulose or PVDF membranes were used for immunoblotting. Bands of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ were detected using mouse monoclonal anti-Ins(1,4,5)$P_3R_1$ KM1112 or mouse monoclonal anti-Ins(1,4,5)$P_3R_2$ KM1083 [26], horse anti-mouse IgG

conjugated to Cy3 as secondary antibody and ECL detection. "SeeBlue" pre-stained standards (4-250 kDa, Novex) were used as molecular weight markers. On 6% SDS-minigels, the myosin standard (250 kDa) ran very close to the Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$.

**Immunolocalisation of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$**

Rat hepatocytes grown on collagen-coated coverslips were rinsed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 30 min, rinsed with PBS, then permeabilised with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. Cells were again rinsed with PBS, blocked with 10% (w/v) fetal bovine serum in PBS for 1 h at room temperature, washed with PBS containing 0.05% (v/v) Tween-20 (PBS-T), and incubated overnight at 4°C with either mouse monoclonal anti-Ins(1,4,5)$P_3R_1$ KM1112 or mouse monoclonal anti-Ins(1,4,5)$P_3R_2$ KM1083 [26]. The cells were then washed with PBS-T and incubated for 1 h at room temperature in the dark with the secondary antibody, Cy3-conjugated anti-mouse IgG, at 1:1000 dilution in PBS-T containing 1% (v/v) serum. After washing with PBS-T, then four times in PBS, the coverslips were mounted on glass slides and viewed using a BioRad MRC-1000 confocal microscope, Krypton-Argon laser, and Chroma 31002 (excitation 515-550 nm, emission 575-615 nm) filters, and x60 oil objective. The location of the ER in fixed freshly-isolated rat hepatocytes in primary culture was determined using $DiOC_6(3)$ and confocal microscopy as described previously [9].

Biochemical Journal Immediate Publication. Published on 28 May 2004 as manuscript BJ20040637



## RESULTS

### Effects of Ins(1,4,6)$P_3$, Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$ on $Ca^{2+}$ inflow and release

The strategy employed was to use Ins(1,4,5)$P_3$ analogues with different affinities for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ as probes for the involvement of each of these receptor subtypes in the activation of SOCs. The analogues employed were D-*myo*-Ins(1,4,6)$P_3$ (higher affinity for Ins(1,4,5)$P_3R_1$) and D-*myo*-Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$ (higher affinity for Ins(1,4,5)$P_3R_2$) [21]. The abilities of D-*myo*-Ins(1,4,6)$P_3$, Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$, to activate $Ca^{2+}$ release and $Ca^{2+}$ inflow in rat hepatocytes were investigated by microinjecting each analogue into hepatocytes together with fura-2. The cells were incubated in the absence of added $Ca^{2+}{}_o$, then $Ca^{2+}{}_o$ was added (to allow an estimate of the rate of $Ca^{2+}$inflow) followed by vasopressin (to release $Ca^{2+}$ remaining in the intracellular stores). It has previously been shown that each of these analogues is resistant to metabolism by 5'-phosphatase and 3'-kinase activities [31-34]. The results obtained for a high intracellular concentration (estimated to be 220 μM) of Ins(1,3,6)$P_3$ are shown in Figure 1A, and those for a control cell, microinjected with fura-2 alone, in Figure 1B. The amount of $Ca^{2+}$ released from the ER by the Ins(1,4,5)$P_3$ analogue was estimated by determining the difference between the $Ca^{2+}$ released by vasopressin in the control cell and that released by vasopressin in the cell loaded with the Ins(1,4,5)$P_3$ analogue. (The rationale for this procedure is discussed in more detail in Materials and Methods.)

The dose-response curve for the effect of Ins(1,3,6)$P_3$ on $Ca^{2+}$ release and $Ca^{2+}$ inflow is shown in Figure 2A. This shows that the concentration of Ins(1,3,6)$P_3$ which gave half-maximal stimulation of $Ca^{2+}$ inflow is substantially higher than that which gave half-maximal stimulation of $Ca^{2+}$ release. A similar dose-response pattern but with less pronounced differences, was observed with Ins(1,2,4,5)$P_4$ (Figure 2B). D-2-deoxy-Ins(1,4,5)$P_3$ (higher affinity for Ins(1,4,5)$P_3R_2$ than Ins(1,4,5)$P_3R_1$ [21]) was also tested. However, the microinjection of D-2-deoxy-Ins(1,4,5)$P_3$ had no effect on either $Ca^{2+}$ release or $Ca^{2+}$inflow, most likely due to its rapid metabolism in liver cells [35].

In contrast to the results obtained with Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$, when the experiment was conducted with Ins(1,4,6)$P_3$ (Figure 2C), the concentration of Ins(1,4,6)$P_3$ which gave half-maximal stimulation of $Ca^{2+}$ inflow was found to be lower than that which gave half-maximal stimulation of $Ca^{2+}$ release. Thus, relative to the ability to induce $Ca^{2+}$ release, Ins(1,4,6)$P_3$ is more effective than Ins(1,3,6)$P_3$ in inducing $Ca^{2+}$ inflow.

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**Intracellular distribution of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ under conditions employed for the measurement of $Ca^{2+}$ inflow**

The intracellular locations of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ were determined using antibodies specific for these proteins and immunofluorescence. The specificity of the antibodies for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ was confirmed by Western blot analysis using extracts of L15 mouse fibroblasts [27], which express Ins(1,4,5)$P_3R_1$ but do not express significant levels of Ins(1,4,5)$P_3R_2$ [29], and extracts of SaOS-2 human osteoblasts, which predominantly express Ins(1,4,5)$P_3R_2$ and Ins(1,4,5)$P_3R_3$ [28]. A single band corresponding to the expected size of the Ins(1,4,5)$P_3R$ was observed in each case (results not shown). In extracts of rat liver, each antibody also gave a single band corresponding to the expected size of the Ins(1,4,5)$P_3R_1$ or Ins(1,4,5)$P_3R_2$ (results not shown).

In immunofluorescence experiments with hepatocytes cultured for 2 h under conditions similar to those employed for the measurement of $Ca^{2+}$ inflow and release, Ins(1,4,5)$P_3R_1$ was principally located in a band at the cell periphery with little in the interior of the cytoplasmic space, and none in the nucleus (Figure 3A). Ins(1,4,5)$P_3R_2$ was also located in a clearly-defined band at the cell periphery with some expression also in the cytoplasmic space (Figure 3B). In some experiments, labelling of the nucleus by anti-Ins(1,4,5)$P_3R_2$ was seen (results not shown). Panels b, f and j of each of Figures 3A and 3B show equatorial sections from three representative cells. Images were also obtained at 3 μm above (panels c, g, k) and below (panels d, h, l) the equatorial plane (the diameter of the cultured cells in the Z axis was 10-20 μm). More intense staining was observed for the images obtained 3 μm above the equatorial plane (closer to the coverslip on which the cells were attached).

Freshly-isolated hepatocytes exhibit little polarity, but when attached to a collagen-coated glass surface begin to regain polarity (assessed, for example, by formation of the cortical actin cytoskeleton) after about 4 h in culture [15,36]. To determine whether re-polarisation affects the intracellular distribution of Ins(1,4,5)$P_3R$, the distribution of Ins(1,4,5)$P_3R_2$ was determined at 1 and 4 h after the initiation of cell culture. Ins(1,4,5)$P_3R_2$ was studied because it gave a more intense immunofluorescence signal than that generated by Ins(1,4,5)$P_3R_1$. Images obtained in the equatorial plane are shown in Figure 4. There was a significant increase in total immunofluorescence due to Ins(1,4,5)$P_3R_2$ at 4 h, possibly due to increased synthesis and/or decreased degradation of Ins(1,4,5)$P_3R$, although there was no change in the intracellular distribution of Ins(1,4,5)$P_3R_2$ (Figure 4).

The distribution of the ER in hepatocytes cultured under conditions similar to those employed for the measurement of $Ca^{2+}$ inflow was also determined using $DiOC_6(3)$. The results indicate that the ER is distributed throughout the cytoplasmic space and, at the periphery of the cell, extends to the plasma membrane (results not shown, and [15]).

Biochemical Journal Immediate Publication. Published on 28 May 2004 as manuscript BJ20040637



## DISCUSSION

The most interesting aspect of this study is the observation that the relationship between the dose-response curves for $Ca^{2+}$ inflow and $Ca^{2+}$ release for each of Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$ differs from that for Ins(1,4,6)$P_3$. Thus Ins(1,4,6)$P_3$ which, on the basis of studies conducted with Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ expressed in insect Sf9 cells [21], has a higher affinity for Ins(1,4,5)$P_3R_1$ than Ins(1,4,5)$P_3R_2$, activated $Ca^{2+}$ inflow at lower concentrations than those which induced significant $Ca^{2+}$ release. By contrast, Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$, each of which has a higher affinity for Ins(1,4,5)$P_3R_2$ than Ins(1,4,5)$P_3R_1$ [21], induced $Ca^{2+}$ release at lower concentrations than those which activated substantial $Ca^{2+}$ inflow. These results suggest that, in intact hepatocytes, the binding of Ins(1,4,5)$P_3$ to Ins(1,4,5)$P_3R_1$s is more effective in activating SOCs than the binding of Ins(1,4,5)$P_3$ to Ins(1,4,5)$P_3R_2$s.

It is recognised that there are a number of assumptions made in interpretation of the dose-response curves for Ins(1,4,5)$P_3$ analogues reported here. Firstly, the measured difference in apparent affinity of a given analogue for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ is relatively small [21]. Secondly, it is assumed that Ins(1,3,6)$P_3$ and Ins(1,4,6)$P_4$ are resistant to metabolism [33,34] and diffuse throughout the cytoplasmic space [37] so that the cytoplasmic concentration of the Ins(1,4,5)$P_3$ analogue remains approximately constant during the period over which $Ca^{2+}$ inflow and $Ca^{2+}$ release are measured. Thirdly, it was not possible to raise the intracellular concentrations of Ins(1,3,6)$P_3$ and Ins(1,2,4,5)$P_4$ to saturate the Ins(1,4,5)$P_3$R. Fourthly, the amount of $Ca^{2+}$ released was estimated using a somewhat indirect strategy. While these assumptions appear valid, some caution should be exercised in interpreting the dose-response curves. Nevertheless, the difference in the patterns of the dose-response curves for analogues with different affinities for Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ is quite striking.

Previously, the differences in affinities of Ins(1,3,6)$P_3$, Ins(1,4,6)$P_3$ and Ins(1,4,5)$P_3$ for Ins(1,4,5)$P_3$Rs have been rationalised by comparing their molecular structures in diagrams which align the phosphate groups of the three molecules [21,33,38]. Molecular docking experiments using the recently published X-ray crystal structure [39] of the Ins(1,4,5)$P_3R_1$ binding domain can be used to extend this model by suggesting how the relative affinities of Ins(1,4,6)$P_3$ and Ins(1,3,6)$P_3$ for Ins(1,4,5)$P_3R_1$ may be related to their abilities to mimic Ins(1,4,5)$P_3$ in the context of the Ins(1,4,5)$P_3R_1$ binding site (Figure 5). An equivalent crystal structure for an Ins(1,4,5)$P_3R_2$ domain is not yet available, but it is interesting to note that the residues of Ins(1,4,5)$P_3R_1$ that interact with Ins(1,4,5)$P_3$ are all conserved in Ins(1,4,5)$P_3R_2$, with the

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exception that Gly-268 in Ins(1,4,5)$P_3R_1$ is replaced by leucine in the Ins(1,4,5)$P_3R_2$ sequence in both the mouse [40] and rat [41] (reviewed in [42]) Ins(1,4,5)$P_3R_2$ sequences. It is possible that the replacement of glycine with the sterically bulky and hydrophobic leucine residue at this position could influence the local environment at the Ins(1,4,5)$P_3R_2$ binding site, perhaps with consequences for the relative affinities of Ins(1,4,5)$P_3$, Ins(1,4,6)$P_3$, Ins(1,3,6)$P_3$ and also Ins(1,2,4,5)$P_4$.

Both Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ were found to be predominantly located at the periphery of the cell in rat hepatocytes cultured under conditions similar to those employed for the measurement of $Ca^{2+}$ inflow (single cells attached to collagen-coated glass slides and cultured for 2 h). At this stage of culture the cells exhibit little polarity [15,36]. Some co-location of Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ at the periphery of the cell may be due to the formation of Ins(1,4,5)$P_3R_1$/Ins(1,4,5)$P_3R_2$ heterotetramers [44]. It is interesting to note that while Ins(1,4,5)$P_3R_1$ and Ins(1,4,5)$P_3R_2$ were both found concentrated near the plasma membrane in hepatocytes cultured for 2 h, the ER was found distributed throughout the cytoplasmic space. This suggests that, for cells in this *in vitro* condition, the majority of the ER has a very low density of Ins(1,4,5)$P_3R$. Previous histochemical studies with freshly-isolated hepatocytes (unpolarised), hepatocyte couplets (partially polarised) and hepatocytes *in situ* (completely polarised) have shown that Ins(1,4,5)$P_3R_1$ is located principally near the plasma membrane while Ins(1,4,5)$P_3R_2$ is located at the plasma membrane and bile canaliculus [17-19]. Moreover, Ins(1,4,5)$P_3R_2$ located around the bile canaliculus appear to be responsible for the initiation of $Ca^{2+}$ waves [17]. Studies employing subcellular fractionation and electron microscopy have provided clear evidence that in liver cells some Ins(1,4,5)$P_3R_1$ are located in regions of the ER which are closely associated with the plasma membrane through F-actin [18,19]. The present immunofluorescent results showing Ins(1,4,5)$P_3R_1$ at the cell periphery are consistent with these results. Thus, the role of Ins(1,4,5)$P_3R_1$ in apparently selectively activating $Ca^{2+}$ inflow is associated with the location of Ins(1,4,5)$P_3R_1$ close to the plasma membrane.

Taken together, the present results with Ins(1,4,5)$P_3$ analogues selective for either Ins(1,4,5)$P_3R_1$ or Ins(1,4,5)$P_3R_2$, the previous results using an anti-Ins(1,4,5)$P_3R_1$ antibody to inhibit Ins(1,4,5)$P_3$ function and adenophostin A to activate $Ca^{2+}$ inflow [9], and the evidence that some Ins(1,4,5)$P_3R_1$ are closely associated with the plasma membrane [18,19], suggest that, in hepatocytes, the activation of SOCs requires the release of $Ca^{2+}$ from a small region of the ER close to the plasma membrane and enriched in Ins(1,4,5)$P_3R_1$. The requirement for Ins(1,4,5)$P_3R_1$ for the activation of SOCs in hepatocytes may simply reflect enrichment of those

**Biochemical Journal Immediate Publication. Published on 28 May 2004 as manuscript BJ20040637**



putative subregions of the ER involved in the activation of SOCs with Ins(1,4,5)$P_3R_1$. It is hypothesised here that the role of Ins(1,4,5)$P_3R_1$ is solely to mediate release of $Ca^{2+}$ from the ER. At this stage there is no evidence for direct interaction between the Ins(1,4,5)$P_3R_1$ protein and the SOC protein in hepatocytes. *In vivo*, where the activation of SOCs is initiated by Ins(1,4,5)$P_3$ generated by the hormone-induced activation of phospholipase C, additional factors will likely also influence the relative ability of Ins(1,4,5)$P_3$ to activate SOCs and release $Ca^{2+}$from intracellular stores. These include the rate of metabolism of Ins(1,4,5)$P_3$ [6], the effects of $Ca^{2+}$ on the affinity of Ins(1,4,5)$P_3$ for Ins(1,4,5)$P_3$Rs [45] and the locations of hormone receptors and phospholipase C in relation to that of Ins(1,4,5)$P_3$Rs [46,47].

**ACKNOWLEDGMENTS**

We acknowledge financial support from the Wellcome Trust (Programme Grant 060554 to BVLP) and NHMRC Grant 102125 (to GJB and RBG). The provision of monoclonal antibodies KM1112 against Ins(1,4,5)$P_3R_1$ and KM1083 against Ins(1,4,5)$P_3R_2$ by Professor K. Mikoshiba, The University of Tokyo, is gratefully acknowledged. The authors also acknowledge Dr. Jenny Hiscock for conducting the confocal microscopy and Ms. Lee-Anne Addis for preparation of the typescript.

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## LEGENDS TO FIGURES

**Figure 1 Effects of Ins(1,3,6)$P_3$ on $Ca^{2+}$ inflow and the vasopressin-induced increase in $[Ca^{2+}]_{cyt}$ in single rat hepatocytes**

Single hepatocytes were injected with fura-2 plus 220 μM Ins(1,3,6)$P_3$ (estimated intracellular concentration) (**A**) or fura-2 alone (**B**) 10 min before beginning the measurement of fluorescence. Additions of $Ca^{2+}$ (1.5 mM) and vasopressin (40 nM) were made as indicated by the horizontal bars. Each trace is representative of those obtained for 21-97 individual cells from 2-7 separate hepatocyte preparations.

**Figure 2 Comparison of concentration-response curves for the effects of Ins(1,3,6)$P_3$, Ins(1,2,4,5)$P_4$ and Ins(1,4,6)$P_3$ on the release of $Ca^{2+}$ from intracellular stores and on $Ca^{2+}$ inflow**

Ins(1,3,6)$P_3$ (**A**), Ins(1,2,4,5)$P_4$ (**B**) or Ins(1,4,6)$P_3$ (**C**) was co-injected into single hepatocytes with fura-2 10 min before beginning the measurement of fluorescence, and $Ca^{2+}$ and vasopressin were added to the incubation medium as described in Figure 1. Rates of $Ca^{2+}$ inflow (■) and amounts of $Ca^{2+}$ release (●) were estimated as described in Materials and Methods. Each data point is the mean ± S.E.M. of the values obtained from 17-97 individual hepatocytes from 1-7 separate hepatocyte preparations.

**Figure 3 Localisation by immunofluorescence of the type I (A) and type II (B) Ins(1,4,5)$P_3$ receptors in rat hepatocytes grown for 2 h in primary culture**

The culture of rat hepatocytes, permeabilisation and cell fixation, staining with anti-Ins(1,4,5)$P_3$ antibody, and confocal microscopy were performed as described in Materials and Methods. (**A**) Cells stained with mouse monoclonal anti-Ins(1,4,5)$P_3R_1$ antibody KM1112 and anti-mouse antibody conjugated to Cy3. (**B**) Cells stained with mouse monoclonal anti-Ins(1,4,5)$P_3$R2 antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of 3 experiments employing 3 separate rat hepatocyte preparations which each gave similar results. The scale bar represents 10 μm. Panels (a-d), (e-h) and (i-l) each represent a different single cell, showing bright field image, equatorial image, 3 μm above equatorial image (Z plane) and 3 μm below equatorial, respectively. Panels (m) and (n) are controls in which primary antibody has been omitted. All images in **A** and **B** were obtained with the same confocal gain setting.

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**Figure 4 Localisation by immunofluorescence of the type II Ins(1,4,5)$P_3$ receptor in rat hepatocytes grown for 1 and 4 h in primary culture**

The culture of rat hepatocytes, permeabilisation and cell fixation, staining with anti-Ins(1,4,5)$P_3$ antibody, and confocal microscopy were performed as described in Materials and Methods. Cells were stained with mouse monoclonal anti-Ins(1,4,5)$P_3$R2 antibody KM1083 and anti-mouse antibody conjugated to Cy3. The results shown are those obtained for one of 3 experiments employing 3 separate rat hepatocyte preparations which each gave similar results. The scale bar represents 10 μm. Panels a-f represent different cells showing the bright field images (a, c, e) and immunofluorescence (equatorial sections) (b, d, f) for cells cultured at 1 h. Panels h-m represent different cells showing the bright field images (h, j, l) and immunofluorescence (equatorial sections) (i, k, m) for cells cultured at 4 h. Panels g and n are controls in which the primary antibody has been omitted.

**Figure 5 Structure of the Ins(1,4,5)$P_3R_1$ binding site based on the x-ray crystal structure of the mouse Ins(1,4,5)$P_3R_1$ core binding domain**

The crystallographically-observed position of bound Ins(1,4,5)$P_3$ [39] is shown in orange. Molecular docking experiments suggest that Ins(1,4,6)$P_3$ (green) may be a relatively effective mimic of Ins(1,4,5)$P_3$ at the Ins(1,4,5)$P_3R_1$ binding site because it can bind in an orientation that allows its phosphate groups to mimic the three phosphate groups of Ins(1,4,5)$P_3$, while its axial 2-hydroxyl group is accepted by an open region close to Gly-268. However, Ins(1,3,6)$P_3$ (purple) may be prevented from adopting a similar binding mode due to unfavourable steric interactions of its axial 2-hydroxyl group with Arg-568 and Lys-569. Molecular docking experiments were carried out using the X-ray crystal structure of the Ins(1,4,5)$P_3$-binding core of mouse type 1 Ins$P_3$R in complex with Ins(1,4,5)$P_3$ (1N4K [39]) according to methods previously described [43]. For clarity, six molecules of water included in the docking experiments and the hydrogen atoms of Ins(1,4,5)$P_3$, Ins(1,4,6)$P_3$ and Ins(1,3,6)$P_3$ are not shown.

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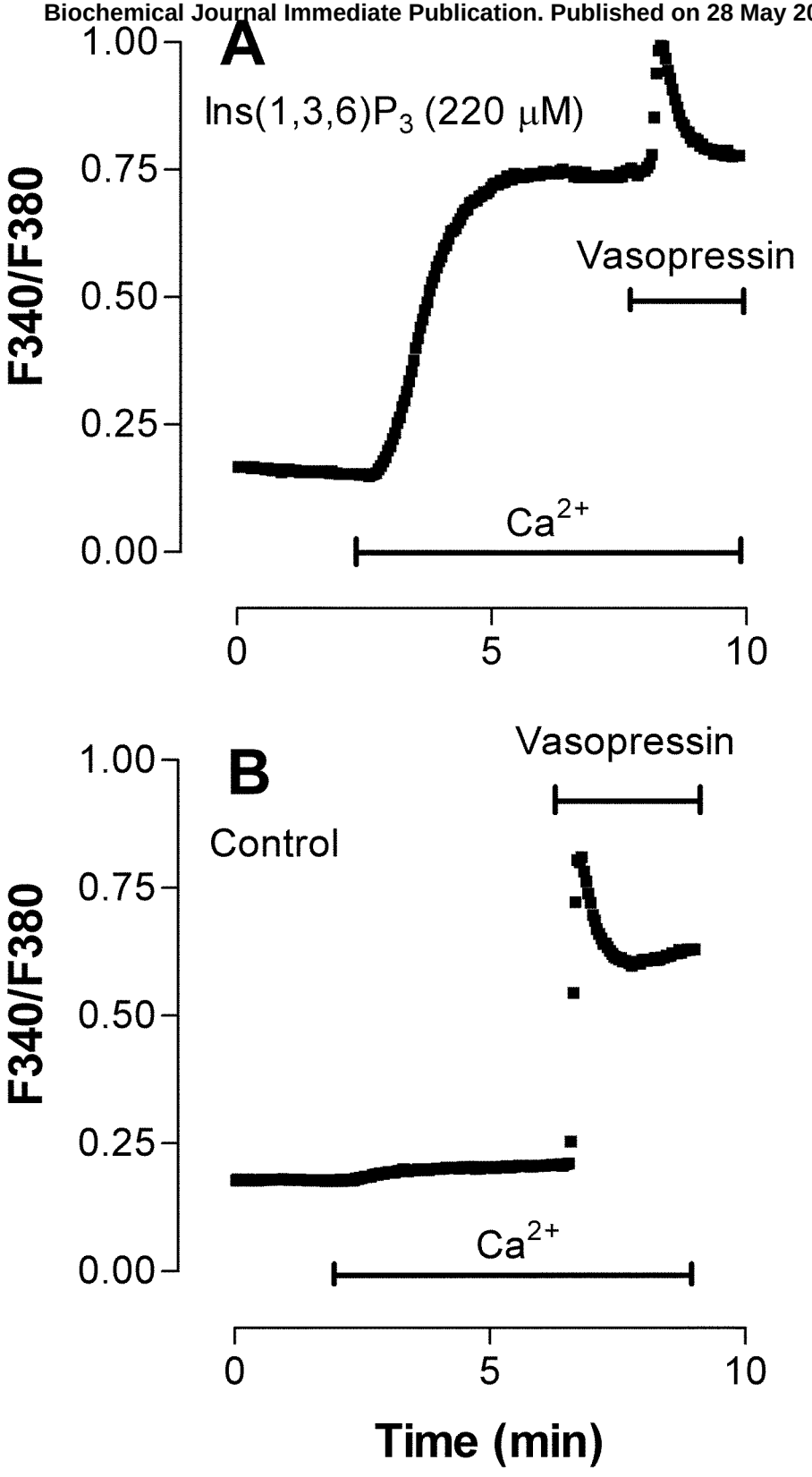


Figure 1

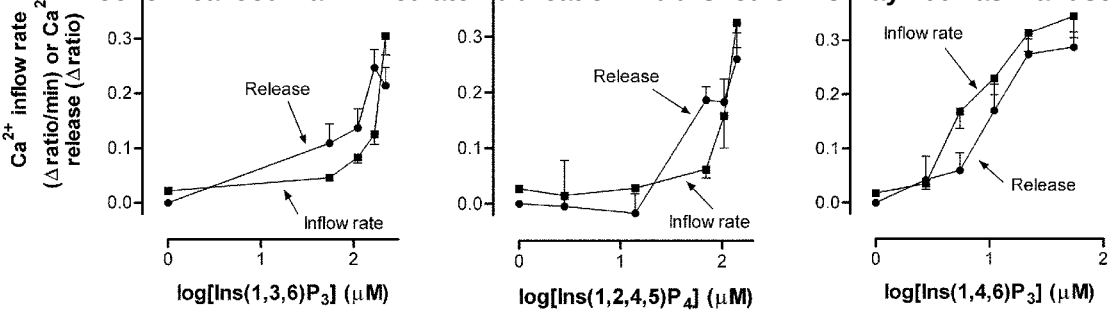




Figure 2

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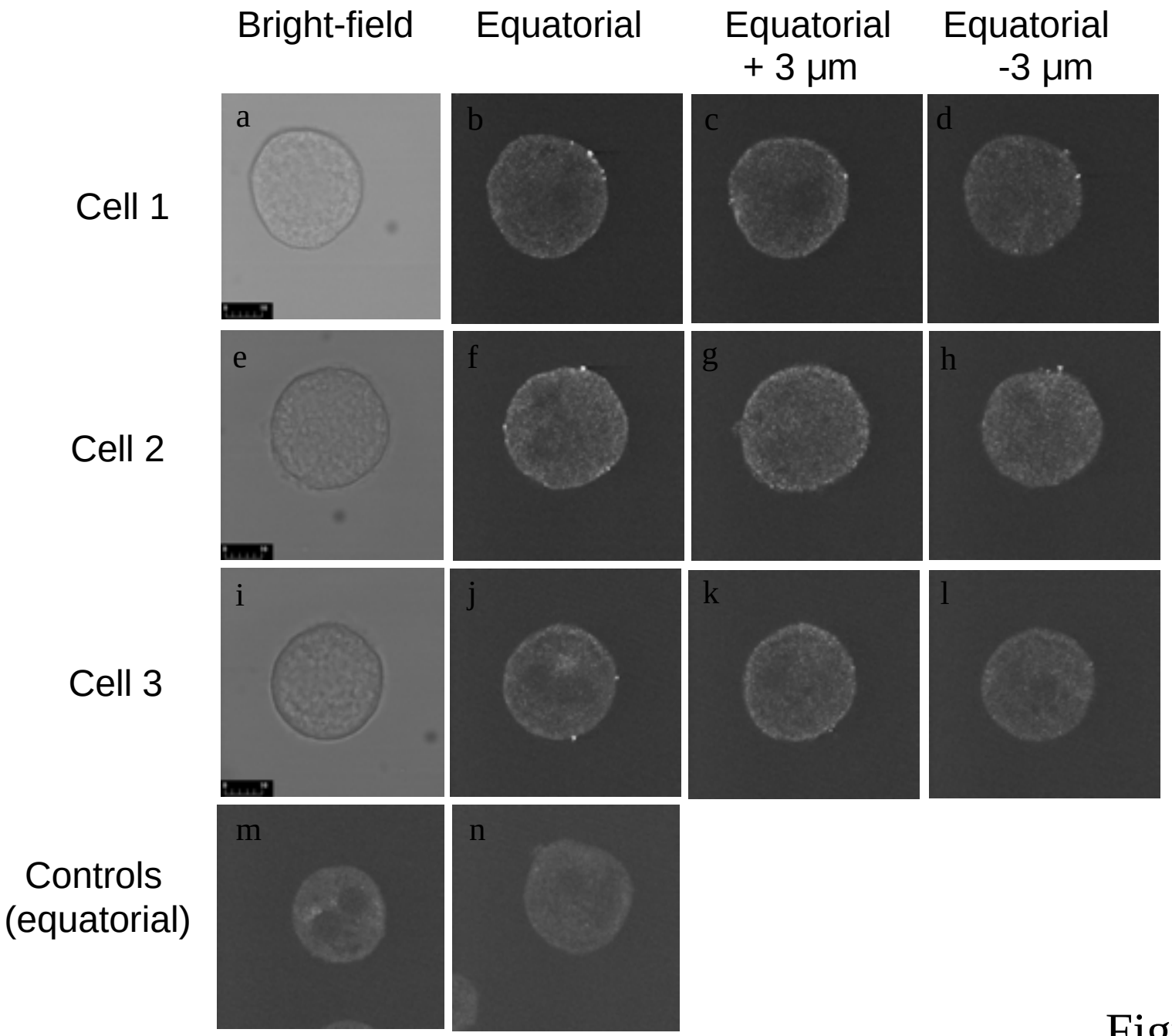


Figure 3A

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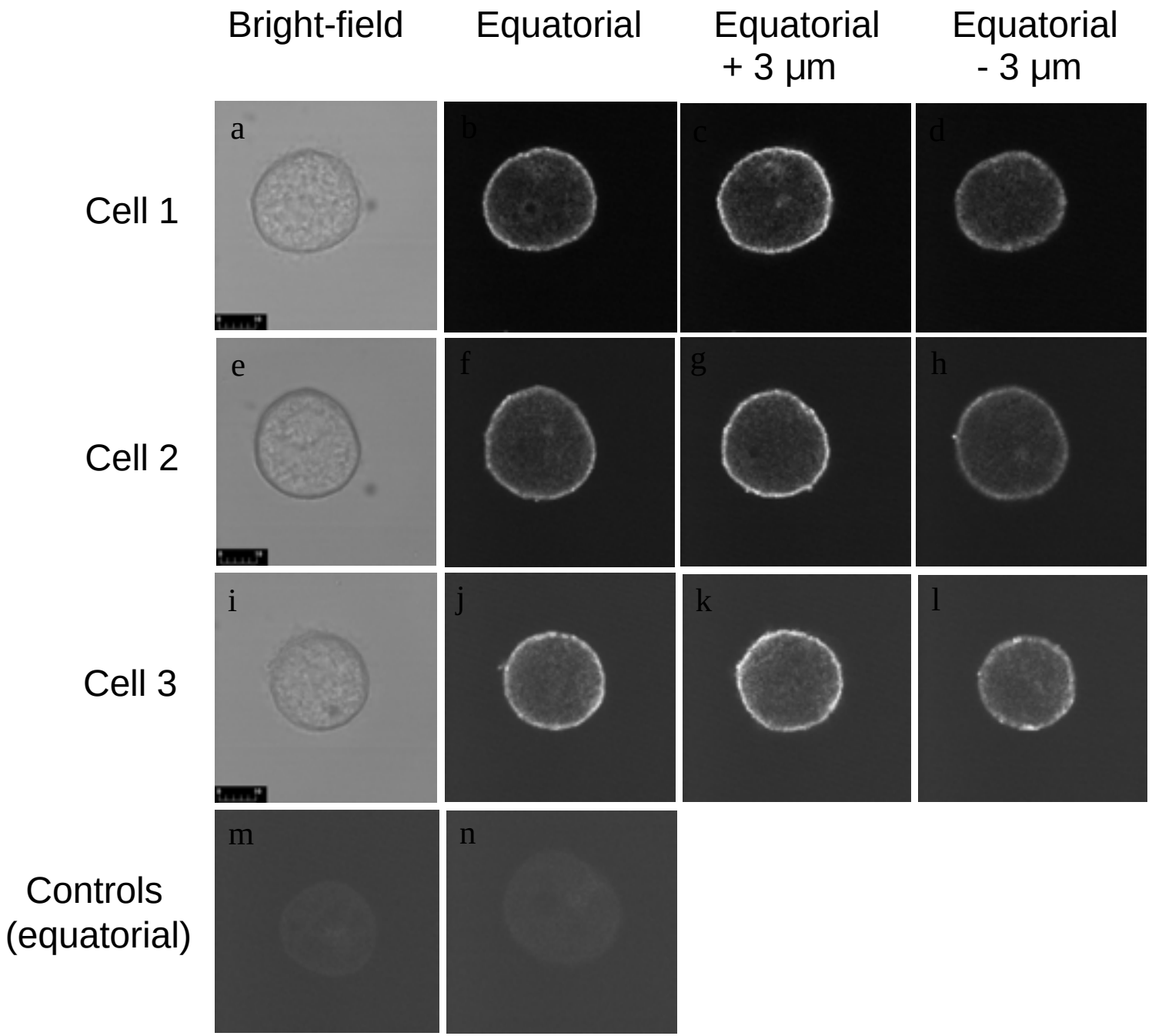


Figure 3B

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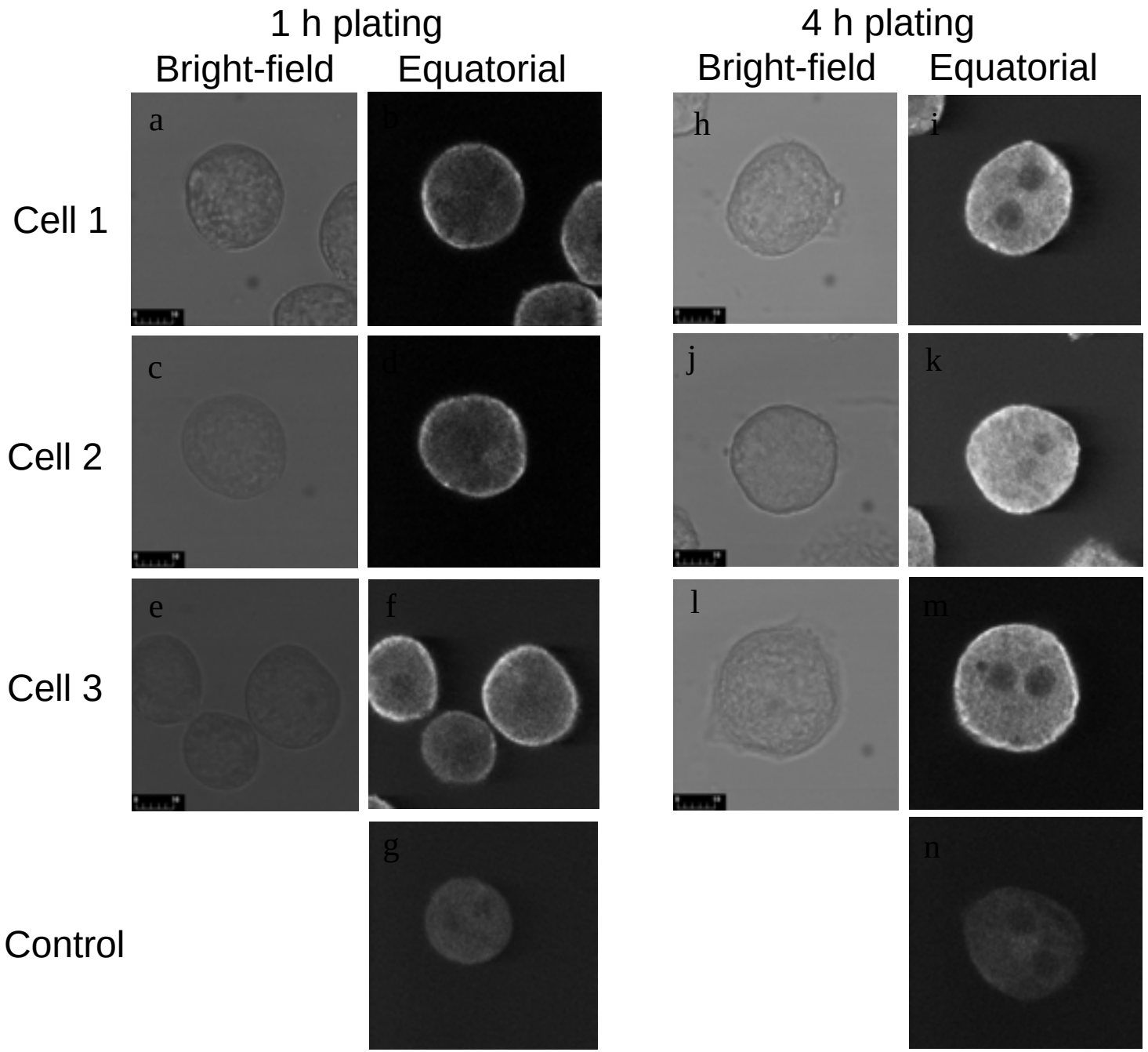


Figure 4

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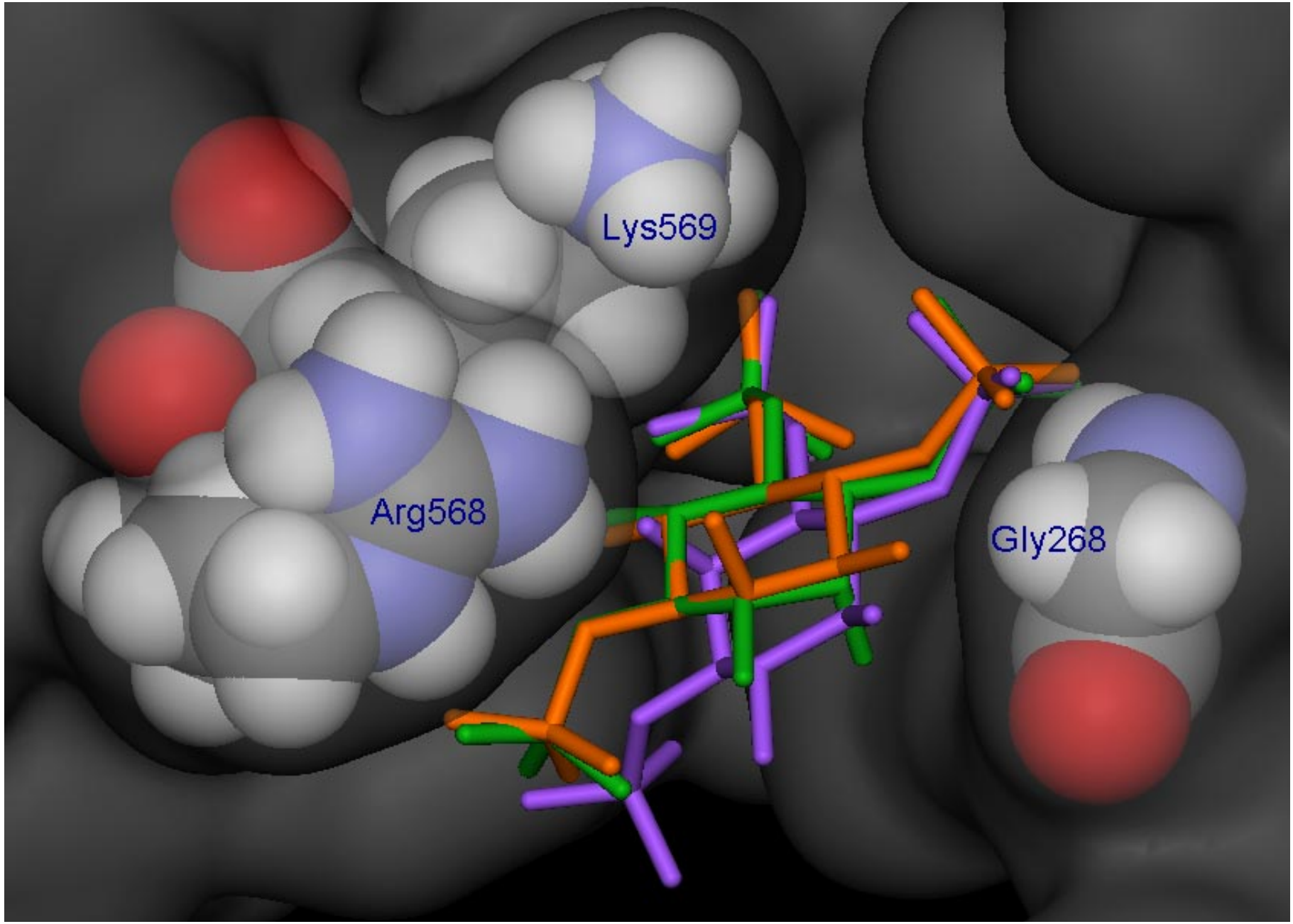


Figure 5